LESSON 19: PROTOPLASTS – METHODS OF ISOLATION

To begin with the Lesson, let us first have a general idea about protoplasts.

Introduction

Protoplasts are plant cells with the cell wall removed. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions, although other sources can be used to advantage.

Two general approaches to removing the cell wall (a difficult task without damaging the protoplast) can be taken—mechanical or enzymatic isolation. Mechanical isolation, although possible, often results in low yields, poor quality and poor performance in culture due to substances released from damaged cells. Enzymatic isolation is usually carried out in a simple salt solution with a high osmoticum, plus the cell wall degrading enzymes. It is usual to use a mix of both cellulase and pectinase enzymes, which must be of high quality and purity. Protoplasts are fragile and easily damaged, and therefore must be cultured carefully. Liquid medium is not agitated and a high osmotic potential is maintained, at least in the initial stages. The liquid medium must be shallow enough to allow aeration in the absence of agitation. Protoplasts can be plated out on to solid medium and callus produced. Whole plants can be regenerated by organogenesis or somatic embryogenesis from this callus. Protoplasts are ideal targets for transformation by a variety of means.

Importance

In plant breeding programmes, many desirable combinations of characters cannot be transmitted through conventional methods of genetic manipulations. Only recently has "another process, other than the sexual cycle, become available for higher plants that can lead to genetic recombination" (Cocking 1979). This non-conventional genetic procedure involving fusion between isolated somatic protoplasts (wall-less naked cells) under in vitro conditions and subsequent development of their product (heterokaryon) to a hybrid plant is known as somatic hybridisation. Since the first report on protoplast fusion-derived somatic hybrid plants of Nicotiana glauca (+) N.langsdorfii by Carlson et al. (1972), somatic hybridisation has opened up several possibilities for the parasexual manipulation of plants.

Protoplast culture provides excellent opportunities for research on plant improvement: first, by exploring genetic variations among the existing crops and then attempting transfer of the available genetic information from one species to another through fusion of protoplasts isolated from somatic tissues of these crops (Razdan and Cocking 1981).

Plastids and mitochondrial genomes (cytoplasmically encoded traits) are inherited maternally in sexual crossings. Through the fusion process the nucleus and cytoplasm of both parents are mixed in the hybrid cell (heterokaryon). This results in various nucleo-cytoplasmic combinations. Sometimes interactions in the plastome and genome contribute to the formation of cybrids (cytoplasmic hybrids). Cybrids, in contrast to conventional hybrids, possess a nuclear genome from only one parent but cytoplasmic genes from both parents. The process of protoplast fusion resulting in the development of cybrids is known as cybridisation. In cybridisation, heterozygosity of extra chromosomal material can be obtained, which has direct application in plant breeding. Although there have been limitations in obtaining novel plants with desirable agronomic traits of many economically important species by somatic hybridisation, or cybridisation, studies during last decade have revealed that the process of protoplast fusion may be a useful tool for the induction of genetic variability and combination of traits which do not exist in nature.

Isolation of Protoplasts

Methods of protoplast isolation can be classified into three main groups:

a. mechanical (non-enzymatic),

b. sequential enzymatic (two-step) and

c. mixed enzymatic (simultaneous) procedures.

Mechanical isolation is done by cutting plasmolysed tissue with a sharp edged knife and releasing the protoplasts by deplasmolysis. The principal deficiency of this approach is that the protoplasts released are few in number; mechanical isolation is thus only of historical importance now. Isolation of protoplasts mechanically from higher plants was pioneered by Klercker in 1892. Generally, protoplasts were isolated from highly vacuolated cells of storage tissues (onion bulbs, scales, radish root, mesocarp of cucumber and beet root).

Cocking (1960) used a concentrated solution of cellulase enzyme (prepared from cultures of the fungus Myrothecium verrucaria to degrade cell walls and demonstrated the possibility of large-scale protoplast isolation from higher plants. Further progress in enzymatic isolation of protoplasts was achieved as soon as cellulase and macerozyme enzymes became available commercially in 1968. Takebe et al. (1968) employed sequential or two-step procedure for isolating mesophyll protoplasts using commercial preparations of enzymes. The sequential approach involves initial incubation of macerated plant tissues with pectinases which, in turn, are then converted into protoplasts by a cellulase treatment. However, Power and Cocking (1968) mixed two enzymes together (simultaneous procedure) and isolated protoplasts in one-step. In this mixed enzymatic approach, plant tissues are plasmolysed in the presence of a mixture of pectinases and cellulases, thus inducing concomitant separation of cells and degradation of their walls to release the protoplasts directly. Most workers use the onestep method because it is less time-consuming and reduces the chances of microbial contamination by eliminating some steps.

The use of commercially available enzymes has enabled the isolation of protoplasts from practically every plant tissue in which cells have not acquired lignification. However, the reproducible potential for regeneration has been observed mostly in protoplasts isolated from leaf mesophyll tissues or cell suspension cultures. Isolation of viable and culturable protoplasts in large quantities is affected by several factors and it is necessary to establish optimum conditions for a system empirically.

Source of Protoplasts

I. Leaves

The leaf is the most convenient and popular source of plant protoplasts because it allows isolation of a large number of relatively uniform cells. Protoplast isolation from leaves involves five basic steps:

a. sterilisation of leaves,

b. removal of epidermal cell layer,

c. pre-enzyme treatment,

d. incubation in enzyme and

e. isolation by filtration and centrifugation

Isolation of protoplasts (Fig.1.) is readily achieved by treating cells/tissues with a suitable mixture of cell wall degrading enzymes. Usually a mixture of pectinase or macerozyme (0.1-1.9%) and cellulase (1-2%) is appropriate for most plant materials.

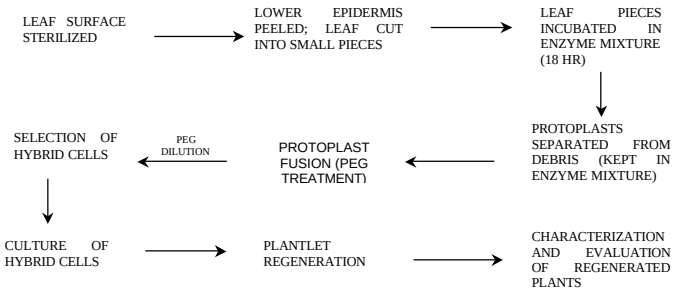
LEAF SURFACE STERILIZED
LOWER EPIDERMIS PEELED; LEAF CUT INTO SMALL PIECES
LEAF PIECES INCUBATED IN ENZYME MIXTURE (18 HR)
PROTOPLASTS SEPARATED FROM DEBRIS (KEPT IN ENZYME MIXTURE)
PROTOPLAST FUSION (PEG TREATMENT)
PEG DILUTION
SELECTION OF HYBRID CELLS
CULTURE OF HYBRID CELLS
PLANTLET REGENERATION
CHARACTERIZATION AND EVALUATION OF REGENERATED PLANTS

Fig. 1. A schematic representation of the various steps in somatic hybridization.

Hemicellulase may be necessary for some tissues. Generally, crude commercial preparations of enzymes are used. The pH of enzyme solution is adjusted between 4.7 and 6.0 and the temperature is kept at 25-30°C. The osmotic concentration of enzyme mixture and of subsequent media is elevated (usually by adding 500-800 m mol/l sorbitol or mannitol) to stabilize the protoplasts and to prevent them from bursting. Usually, 50-100 m mol/l $CaCl_2$ is added to the osmoticum as it improves plasma membrane stability. The cells and tissues are incubated in the enzyme mixture for few to several (generally, 16-18) hours. Naked protoplasts devoid of cell wall are gradually released in the enzyme mixture (Fig. 2.).

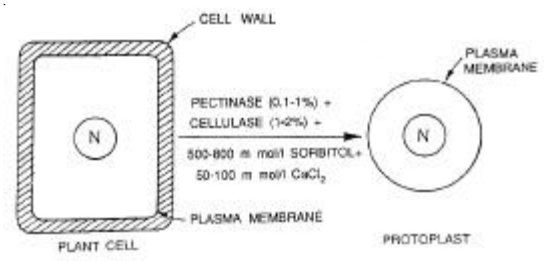

Fig.2. Production of protoplasts by enzyme treatment (enzymes are depicted above the arrow). Osmoticum (shown below the arrow) is added to stabilize the protoplasts and prevent them from bursting.

Protoplasts have been isolated from virtually all plant parts, but leaf mesophyll is the most preferred tissue, at least in case of dicots, for this purpose. In general, fully expanded leaves are surface sterilized, their lower epidermis is peeled off with a pair of forceps and the peeled areas are cut into small (1 cm^2) pieces with a scalpel and suspended in the enzyme mixture. When epidermis can not be peeled (like in monocots), leaf may be cut into 1 mm^2 pieces and treated with the enzyme mixture; vacuum infiltration may be used to facilitate the entry of enzymes into the tissues. As soon as the vacuum is removed, the leaf pieces will sink and eventually release the mesophyll protoplasts. Through this approach a large yield of protoplasts can be obtained in comparatively short periods.

After the period of incubation, protoplasts are washed with a suitable washing medium in order to remove the enzymes and the debris. The protoplasts may be cultured on a suitable medium in a variety of ways:

i. Bergmann's plating technique (in agar medium),

ii. In a thin layer of liquid medium or

iii. In small microdrops of 50-100ul. Protoplasts readily regenerate cell wall (within 2-4 days) and undergo mitosis to form macroscopic colonies which can be induced to regenerate whole plants. The conditions for isolation and culture of protoplasts and regeneration of complete plants has been standardized for a large number of plant species, but cereals still present some problems.

Generally, MS and B5 media, and their modifications are used for protoplast culture. The media are supplemented with a suitable osmoticum and, almost always, with an auxin and a cytokinin, their types and concentrations depending mainly on the plant species. After 7-10 days of culture, protoplasts regenerate cell wall and the osmolarity of medium is gradually reduced to that of normal medium. The macroscopic colonies are transferred onto normal tissue culture media. Protoplasts are very sensitive to light, therefore, they are cultured in diffuse light or dark for the first 4-7 days.

II. Callus Cultures

Young callus cultures are also ideal material for obtaining large quantities of protoplasts. Older callus cultures tend to form giant cells with thick cell walls which are usually difficult to digest. Therefore, young actively growing callus is subcultured and used after two weeks for protoplast isolation.

Plant regeneration from cells isolated from cultured tissues could have substantial application in agriculture. Plants regenerated from populations of single cells may retain all the essential characters of a cultivar or clone but selectively alter undesirable genes. For example, sugarcane plants derived from callus cells have been demonstrated to provide clones having all the characteristics of the parent cultivar but for some improved traits such as disease resistance, enhanced yield potential and higher sugar content (Liu and Chen 1978). Isolation of protoplasts from callus tissues is the most recent refinement of single-cell regeneration efforts which have resulted in obtaining variant protoclones (protoplast propagated clones) in agriculturally important species.

III Cell Suspension Cultures

Cell suspension cultures also provide excellent source materials for isolating protoplasts. A high-density cell suspension is centrifuged. After removing the supernatant, cells are incubated in an enzyme mixture (cellulase + pectinase) in a culture flask placed on a platform shaker for 6 hr to overnight depending on the concentration of enzymes. A lower concentration of enzymes helps to prevent the formation of aggregates in the cell suspension in order to obtain better yields.

Protoplasts originating from cell suspension cultures have demonstrated the potentiality to regenerate plants in species in which attempts to induce sustained division of mesophyll protoplasts have failed. Recent success in formation of plantlets from protoplasts of morphogenetic cell culture origin in cereals (pearl millet, sorghum and barley cv. Golden Promise) proved cell suspension cultures are a viable source of totipotent protoplasts.

IV Preconditioned Plant Materials

Mesophyll protoplasts of some crop plants have a low morphogenetic response. This is because of the fact that the physiological state of growth of a donor plant under natural conditions largely affects the regeneration potential of protoplasts in these systems. On the contrary, tissue culture regenerated plants are maintained under uniform physiological conditions and, therefore, provide materials (leaf) preconditioned for protoplast isolation and regeneration. This approach is particularly essential for regeneration of potato protoplasts. Fully expanded leaves excised from 3- to 4-week-old plants raised from virus-free meristem-tip cultures provided a source of highyielding, viable and regenerating protoplasts of tetraploid (2n = 4x = 48) and dihaploid (2n = 2x = 24) potato (Solanum tuberosum) cultivars and related wild species (S. chacoense). In other crops (amphidiploid Brassica napus cv. Victor tomato) also, the in vitro propagated shoot cultures provide protoplasts that regenerated several hundred colonies used for studies in morphogenesis and DNA virus investigation

Preparation of Cytoplasts and Miniprotoplasts

The immediate product of protoplast fusion is a binucleated cell (heterokaryon) with mixed cytoplasm of both parents. During fusion experiments, particularly at interspecific and intergeneric levels, some of the problems associated with the combination of both nuclear genomes and mixed cytoplasmic materials can be overcome by replacing one or even both fusion partners by subprotoplasts (protoplast fragments). Another approach to achieve a more defined combination of nuclear and cytoplasmic materials is to inactivate the nuclear genome of one of the fusion partners by irradiation in order to produce "physiological" subprotoplasts. The ripening pericarp of some solanaceous species produce subprotoplasts naturally whereas in vitro they are formed by 'budding" of protoplasts or by elongation of the protoplasts during plasmolysis. Subprotoplasts can be collected as a distinct fraction from protoplast preparations.

Isolated protoplasts can be experimentally induced to fragment into types of subprotoplasts called miniprotoplasts or cytoplasts. The term miniprotoplast was coined by Wallin et al. (1978) for subprotoplasts having nuclear material. Miniprotoplasts can divide and may be able to regenerate into plants as in Solanum niger (Lesney et al. 1986). Equivalent terms given to miniprotoplasts are karyoplast (evacuolated subprotoplast) or nucleoplast. Cytoplasts are nuclear-free subprotoplasts which do not divide but are useful in the process of cybridisation. The fragmentation of protoplasts is achieved through application of different centrifugal forces created by discontinuous gradients during the process of centrifugation. Additional exposure of isolated protoplasts to cytochalasin B in combination with centrifugation has also been found beneficial for enucleation. Protoplasts are dramatically deformed and 'drawn out' due to centrifugal forces during centrifugation. Different densities of the cellular components then allow the enucleation of protoplasts in iso-osmotic density gradients. Components of high density (nucleus) are oriented toward the bottom and those having less density (vacuoles) move to the top of the centrifuge tube. After prolonged centrifugation either the nuclei surrounded by some cytoplasmic material are pinched off, giving rise to nucleated miniprotoplasts or cytoplasmic materials without nuclei form enucleated cytoplasts. Both types of subprotoplasts eventually become spherical after removal from the gradient.

The composition of the gradient, speed of centrifugation and treatment with cytochalasin B depend on the specific type of protoplast used for enucleation. A broad framework within which a procedure can be developed for a particular protoplast system has been outlined by Lorz (1984) and other workers. Suitable components for establishing gradients for protoplast centrifugation are inorganic salts, sugars and modified silica gels (percoll). Different solutions are gently layered without mixing in the centrifuge tube to form a discontinuous gradient. Centrifugation is performed in a swinging-bucket rotor containing 10 ml tubes (each loaded with 2-5 x 105 protoplasts), using a speed between 20,000 and 40,000 g for 15-20 min at 37°C; or 45-90 min at 12°C. After centrifugation, enucleated cytoplasts are located in the top fraction of the gradient and nucleated miniprotoplasts form a band between layers I and II. Both subprotoplast fractions are collected by Pasteur pipettes, resuspended in the medium and used in the fusion process.

Viability of Protoplasts

Isolated protoplasts should be healthy and viable in order to undergo sustained divisions and regeneration. Methods used to check cell/protoplast viability include:

a. observation of cyclosis or cytoplasmic streaming

b. oxygen uptake by protoplasts measured by an oxygen electrode

c. photosynthetic activity.

These tests indicate active metabolism in protoplasts. Exclusion of Evan's blue dye by intact membranes and staining with FDA are some of the common methods used to determine the viability of freshly isolated protoplasts.

Conclusion

Protoplast culture provides excellent opportunities for research on plant improvement. First, by exploring genetic variations among the existing crops and then, attempting transfer of the available genetic information from one species to another through fusion of protoplasts isolated from somatic tissues of these crops. Leaves are the most convenient and popular source of plant protoplasts as it allows isolation of a large number of relatively uniform cells. This Lesson gives a com1plete account of isolation of protoplasts in a comprehensive manner for a better understanding.

Questions

1. How will you isolate protoplasts from the plant cells?
2. What are the various sources to obtain protoplasts?
3. Give a schematic representation of the steps followed in somatic hybridization.
4. How will you check the viability of protoplasts?

Note